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**{Exhibit 23}**

**Boguslaski et al., U.S. Patent 4,134,792, issued  
January 16, 1979**

## United States Patent [19]

Boguslaski et al.

[11] 4,134,792

[45] Jan. 16, 1979

[54] SPECIFIC BINDING ASSAY WITH AN  
ENZYME MODULATOR AS A LABELING  
SUBSTANCE[75] Inventors: Robert C. Boguslaski; Robert J.  
Carrieco, both of Elkhart, Ind.; James  
E. Christner, Ann Arbor, Mich.[73] Assignee: Miles Laboratories, Inc., Elkhart,  
Ind.

[21] Appl. No.: 748,005

[22] Filed: Dec. 6, 1976

[51] Int. Cl.<sup>2</sup> ..... G01N 33/00; G01N 31/14[52] U.S. Cl. .... 195/99; 195/103.5 A;  
424/12[58] Field of Search ..... 195/103.5 A, 99;  
424/12

## [56] References Cited

## U.S. PATENT DOCUMENTS

3,817,837	6/1974	Rubenstein et al. ....	195/103.5 A
3,850,752	11/1974	Schuurs et al. ....	195/103.5 A
3,880,934	4/1975	Rammler .....	195/103.5 A
3,975,342	8/1976	Gross .....	424/12
4,003,792	1/1977	Mill et al. ....	424/12

## OTHER PUBLICATIONS

Miles et al., *Nature*, vol. 219, (1968), pp. 186-189.  
Landon, "A Suggested Terminology for Non-IsotopicImmunoassays", Conference on Non-isotopic Immuno-  
assays, (1976).

Primary Examiner—Alvin E. Tanenholtz

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Attorney, Agent, or Firm—Andrew L. Klawitter

## [57] ABSTRACT

A specific binding assay method employing, as a labeling substance, a reversibly binding enzyme modulator for the detection of a ligand in a liquid medium. The method follows conventional specific binding assay techniques of either the homogeneous or heterogeneous type wherein the liquid medium to be assayed is combined with reagent means that includes a labeled conjugate to form a binding reaction system having a bound-species and a free-species of the conjugate. The amount of conjugate resulting in the bound-species or the free-species is a function of the amount of ligand present in the liquid medium assayed. In the present invention, the labeled conjugate comprises a reversibly binding enzyme modulator covalently linked to a binding component of the binding reaction system. The distribution of the conjugate between the bound-species and the free-species is determined by addition of an enzyme whose activity is affected, either decreased or increased, by said modulator and measuring the resulting activity of the enzyme. The enzyme modulator may be a conventional enzyme inhibitor, preferably of the competitive type, or an allosteric effector.

19 Claims, 2 Drawing Figures

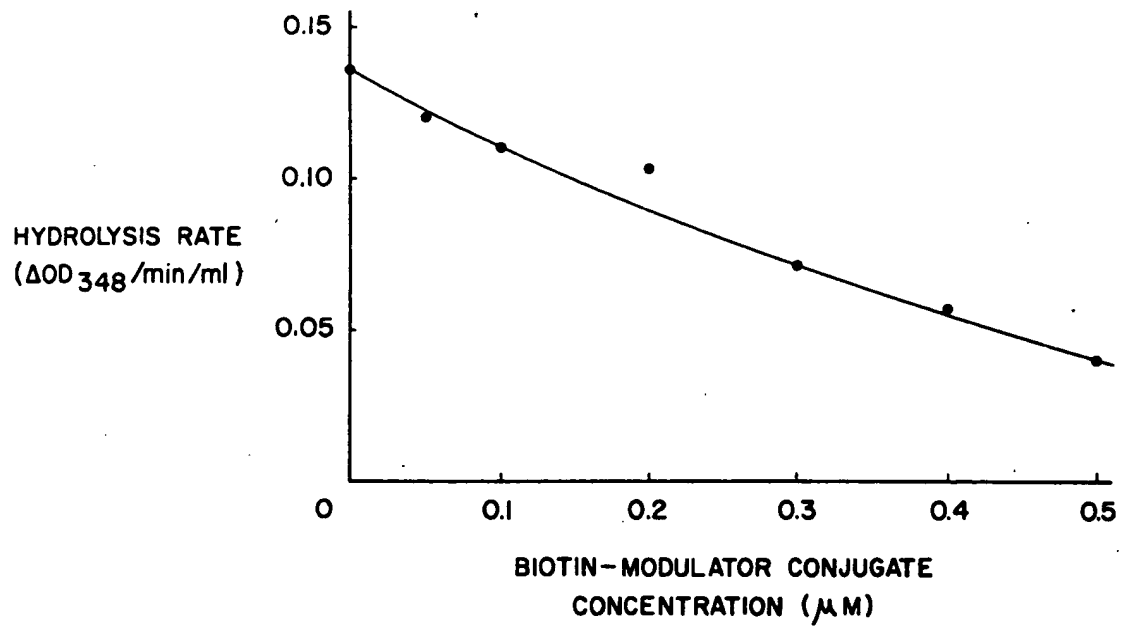


FIG. 1

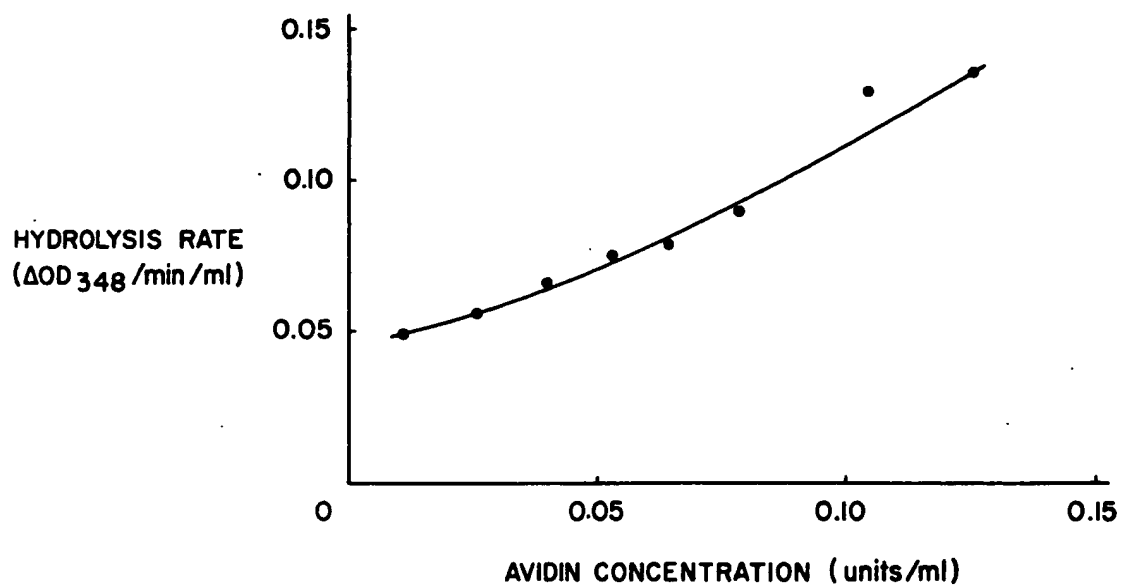


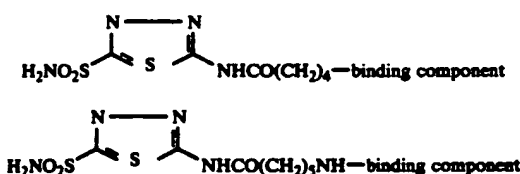
FIG. 2

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active coupling groups at opposite ends to covalently bind to the respective moieties to be linked. The bridge group usually comprises between 1 and 50, and preferably between 1 and 10, carbon atoms or heteroatoms such as nitrogen, oxygen, sulfur, phosphorus, and so forth. Examples of a bridge group comprising a single at  $m$  would be a methylene group (one carbon atom in the chain) and an amino group (one heteroatom in the chain). The bridge group usually has a molecular weight not exceeding 1000 and preferably less than 200. The bridge group, if present, comprises a chain of carbon atoms and/or heteroatoms and is joined to the enzyme modulator and the binding component, respectively, by connecting groups, usually selected from ester, amide, ether, thioester, acetal, methylene, and amino groups.

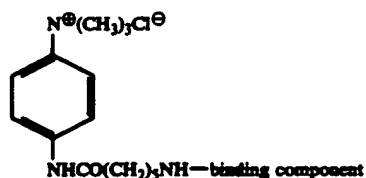
Following are structural formulae of contemplated labeled conjugates including the competitive inhibitors previously listed as the labeling substance and a general method for their preparation.

A. Acetazolamide - binding component conjugates

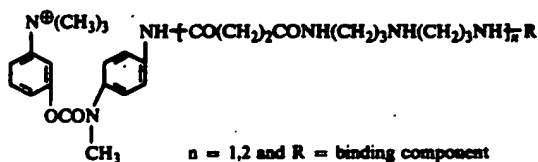


Example 1, Parts B and C provide details of preparations of these types of conjugates.

B. Phenyl trimethylammonium ion - binding component conjugates



The binding component of interest is covalently linked to the amino function of an o-aminoalkyl carboxylic acid through a reaction such as nucleophilic displacement. The resulting product is then coupled to p-amino-N,N-dimethylaniline via an activating agent, such as carbodiimide. The resulting product is then treated with methyl iodide to give the desired conjugate.

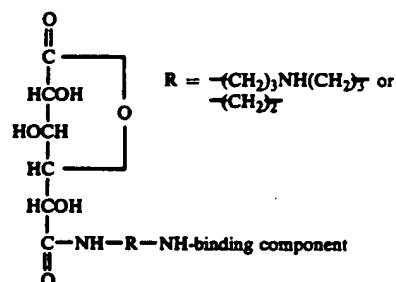


The binding component of interest is coupled through any appropriate means (activation of the carboxyl group, nucleophilic displacement) to one amino group of 3,3'-diaminodipropylamine. The resulting product is then treated with succinic anhydride. The resulting material is then coupled to the free amino function of the N-methyl-N-(p-aminophenyl) carbamate ester of m-(triethylamino)-phenol via a carboxylic acid activating agent. Alternatively, the inhibitor could be ex-

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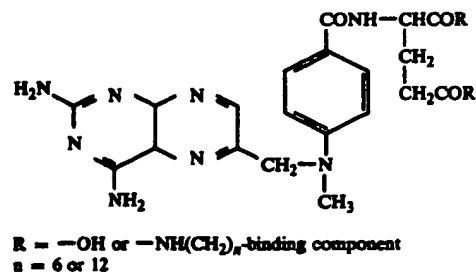
tended further from the ligand by treatment of the above succinylated derivative with 3,3'-diaminodipropylamine and carbodiimide, followed by succinic anhydride and coupling to the inhibitor ( $n=2$ ).

C. Saccharo-1,4-lactone - binding component conjugate



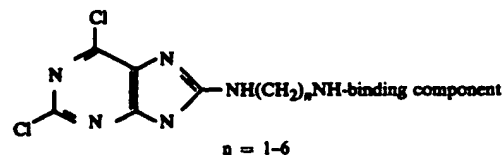
Saccharo-1,4-lactone is coupled to an  $\alpha,\omega$ -diaminoalkyl derivative through its free carboxyl group using a carboxylic acid activating agent. The resulting product is then coupled to the binding component of interest using any appropriate reaction, e.g., nucleophilic displacement, carboxylic acid activation, etc.

D. 4-Amino-10-methylpteroylglutamic acid - binding component conjugate



The binding component of interest is coupled to amino function of an  $\alpha,\omega$ -diaminoalkyl derivative through an appropriate reaction sequence. The product from this reaction is then coupled to one of the carboxylic acid functions of 4-amino-10-methyl pteroyl glutamic acid via an appropriate carboxylic acid activating agent, e.g., carbodiimide.

E. 2,6,8-Trichloropurine - binding component conjugate



2,6,8-Trichloropurine is treated with dihydropyran and a catalytic amount of acid to produce a 9-substituted tetrahydropyranyl ether. This product is then reacted with an  $\alpha,\omega$ -diaminoalkane to produce a C-8 substituted aminoalkylamine derivative. The tetrahydropyranyl blocking group is then removed under acidic conditions to give a 2,6-dichloro-substituted purine. The binding component of interest is then coupled to the free alkyl amino group through any appropriate reaction sequence.

## SPECIFIC BINDING ASSAY WITH AN ENZYME MODULATOR AS A LABELING SUBSTANCE

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

This invention relates to assay methods of the homogeneous and heterogeneous specific binding type for determining qualitatively or quantitatively a ligand in a liquid medium. In a preferred embodiment, the present invention relates to competitive binding immunoassays employing nonisotopic labels.

#### 2. Description of the Prior Art

In conventional specific binding assay techniques, the test sample is combined with reagent means of various compositions that include a conjugate of a labeling substance linked to a binding component which participates with other constituents, if any, of the reagent means to form a binding reaction system producing two species of the labeled conjugate, a bound-species and a free-species. The relative amounts of the labeled conjugate that result in the bound-species and the free-species are a function of the presence or amount of the ligand to be detected in the test sample.

As an illustration, a conventional competitive binding assay technique will now be described. In such a technique the reagent means would comprise (1) a labeled conjugate in the form of the ligand to be detected, or of an appropriate analog thereof, chemically linked to a labeling substance and (2) a specific binding partner for the ligand, such as an antibody or other binding protein. Upon combination of the test sample and the reagent means, the ligand to be detected and the labeled conjugate would compete in a substantially nondiscriminating manner for noncovalent binding to the specific binding partner. As a result, the amount of labeled conjugate that would become bound to the binding partner, i.e., in the bound-species, or that would remain free, i.e., unbound to the binding partner, and, thus, in the free-species, is a function of the amount of competing ligand present.

Where the labeled conjugate in the bound-species and that in the free-species are essentially indistinguishable by the means used to measure the labeling substance, the bound-species and the free-species must be physically separated in order to complete the assay. This type of assay is referred to as "heterogeneous." Where the bound-species and free-species forms of the labeled conjugate can be distinguished, a "homogeneous" format may be followed and the separation step avoided.

The first discovered type of specific binding assay was the radioimmunoassay which employs a radioactive isotope as the labeling substance. Such an assay necessarily must follow the heterogeneous format. Because of the hazard and difficulty of handling radioactive materials, many new assay systems have been devised using materials other than isotopes as the labeling substance, including enzymes, fluorescent molecules, bacteriophages, coenzymes, and luminescent molecules.

The following describe several different heterogeneous binding reaction systems in which an enzyme is employed as the labeling substance: U.S. Pat. Nos. 3,654,909; 3,791,932; 3,839,153; 3,850,752; and 3,879,262; *J. Immunol. Methods* 1:247 (1972); and *J. Immunol.* 109:129(1972). A heterogeneous binding assay utilizing a non-active precursor of a spectrophotometrically detectable substance as the labeling substance is suggested in U.S. Pat. No. 3,880,934. Of fur-

ther background interest pertaining to heterogeneous assays is *Principles of Competitive Protein-Binding Assays*, ed. Odell and Daughaday (J. B. Lippincott Co., Philadelphia, 1972). An enzyme-labeled immunoassay of the homogeneous type is described in U.S. Pat. No. 3,817,834 wherein a ligand-enzyme conjugate is employed. The enzymatic activity of the conjugate in the bound-species is measurably less than that in the free-species, thereby allowing a homogeneous format to be used. The use of particularly unique materials as labeling substances, including coenzymes, luminescent molecules, and cleavable fluorescent substrates, in both homogeneous and heterogeneous formats, is described in U.S. patent applications Ser. Nos. 667,982 and 667,996, filed on Mar. 18, 1976, and assigned to the instant assignee.

It is a primary object of the present invention to provide a specific binding assay method, useable in both the homogeneous and heterogeneous modes, which employs a unique labeling substance belonging to a large class of compounds for which there is an extensive literature background to facilitate selection of specific labels, approaches to derivatization, and monitoring reactions.

### SUMMARY OF THE INVENTION

The present invention provides a specific binding assay method employing, as a labeling substance, a reversibly binding enzyme modulator. The inventive labeling substance may be used in both homogeneous and heterogeneous binding assay formats wherein the liquid medium to be assayed for a ligand is combined with reagent means which includes a labeled conjugate to form a binding reaction system having a bound-species and a free-species of the conjugate. The amount of conjugate resulting in the bound-species or the free-species is a function of the presence or amount of ligand present in the liquid medium assayed. In the present invention, the labeled conjugate comprises a reversibly binding enzyme modulator covalently linked to a binding component of the binding reaction system. The distribution of the conjugate between the bound-species and the free-species is determined by addition of an enzyme whose activity is affected, either in an inhibitory or stimulatory manner, by said modulator and measuring the resulting activity of the enzyme following the desired homogeneous or heterogeneous assay scheme.

### BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a graphic representation of the inhibitory effect of various concentrations of biotin-modulator conjugate on the esterase activity of carbonic anhydrase measured by hydrolysis rate; and

FIG. 2 is a graphic representation of the effect of various concentrations of avidin on the inhibitory effect of biotin-modulator conjugate on the esterase activity of carbonic anhydrase measured by hydrolysis rate.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the context of this disclosure, the following terms shall be defined as follows: "ligand" is the substance, or group of substances, whose presence or the amount thereof in a liquid medium is to be determined; "specific binding partner of the ligand" is any substance, or group of substances, which has a specific binding affinity for the ligand to the exclusion of other substances; "specific binding analog of the ligand" is any substance, or group

excess of that capable of being displaced by all of the ligand in the liquid medium in the time that the specific binding partner, or complex, and the medium are in contact prior to the completion of the assessment of any change in the activity of the conjugated modulator.

Another variation of the competitive binding homogeneous technique is the sequential saturation homogeneous technique wherein the components of the specific binding reaction are the same as those used in the competitive binding technique, but the order of addition or combination of the components and the relative amounts thereof used are different. Following a sequential saturation technique, the specific binding partner of the ligand is contacted with the liquid medium suspected of containing the ligand for a period of time prior to the contact of said liquid medium with the conjugate. The amount of the specific binding partner contacted with the liquid medium is usually in excess of that capable of binding with all of the ligand thought to be present in the liquid medium in the time that the specific binding partner and the liquid medium are in contact prior to the time that the liquid medium is contacted with the conjugate. Further, the amount of the ligand or ligand analog in conjugated form is usually in excess of that capable of binding with the remaining unbound amount of the specific binding partner during the time that the liquid medium and the conjugate are in contact prior to the completion of the assessment of any change in activity of the conjugated modulator.

#### HETEROGENEOUS SCHEMES

The use of an enzyme modulator as labeling substance can also be applied to the conventional heterogeneous type assay schemes wherein the bound- and free-species of the labeled constituent are separated and the quantity of labeling substance in one and/or the other is determined. The reagent means for performing such a heterogeneous assay may take on many different forms. In general, such means comprises three basic constituents, which are (1) the ligand to be detected, (2) a specific binding partner of the ligand, and (3) a labeled constituent which is normally a labeled form of (a) the ligand, (b) a specific binding analog of the ligand, or (c) the specific binding partner. The binding reaction constituents are combined simultaneously or in a series of additions, and with an appropriate incubation period or periods, the labeled constituent becomes bound to its corresponding competing binding partners such that the extent of binding, i.e., the ratio of the amount of labeled constituent bound to a binding partner to that unbound, is a function of the amount of ligand present. To follow is a brief description of some of the different heterogeneous binding reaction schemes that may be used in carrying out the method of the present invention.

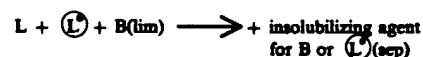
For the diagrams which are set out hereinafter, the following legend shall apply:

Symbol	Definition
$\odot$	ligand to be detected in sample
$\odot$	ligand or specific binding analog thereof
B	binding partner for the ligand
*	labeling substance, i.e., enzyme modulator
$\vdash$	insoluble phase
$\longrightarrow$	incubation period
(sep)	appropriate separation of the

-continued

Symbol	Definition
(lim)	bound- and free-species limited; present in an amount less than that capable of being bound to the total available binding sites under the selected reaction conditions during the selected incubation period; i.e., the constituent for which the other constituents compete for binding
(exc)	excess; present in an amount greater than that capable of being bound by the total available binding sites under the selected reaction conditions during the selected incubation period

#### 1. Competitive binding heterogeneous formats



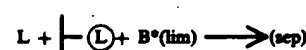
This is the classical competitive binding approach. Examples of insolubilizing agents are specific precipitating antibodies, specific insolubilized antibodies, and, where B or  $\odot$  is a proteinaceous material, protein precipitators such as ammonium sulfate, or where B or  $\odot$  is a small adsorbable molecule, dextran-coated charcoal. Description of parallel systems may be found in *Biochem. J.* 88:137 (1963) and U.S. Pat. No. 3,839,153.



This approach is commonly referred to as the solid-phase technique. Descriptions of parallel radioimmunoassay and enzyme immunoassay techniques may be found in U.S. Pat. Nos. 3,505,019; 3,555,143; 3,646,346; and 3,654,090.

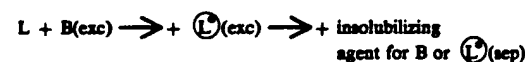


Reference: U.S. Pat. No. 3,654,090.

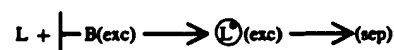


Reference: U.S. Pat. No. 3,850,752.

#### 2. Sequential saturation heterogeneous formats



In the sequential saturation technique, some or all the binding sites on B remaining after the first incubation period are bound by the labeled constituent.



Descriptions of parallel radioimmunoassay and enzyme immunoassay techniques may be found in U.S. Pat. N. 3,720,760 and *J. Immunol.* 209:129(1972).

stirred for 1 hr at 0° C. with 100 ml 1.5 N hydrochloric acid and the resulting yellow precipitate was collected by filtration and stirred for 1 hr at 0° C. with 100 ml 10% (by weight) sodium hydroxide. The yellow solid was collected by filtration and recrystallized from aqueous methanol to give 50 mg of yellow solid (9% yield, m.p. 229°-233° C.).

Analysis calculated for  $C_{14}H_{17}N_7O_7S_2$ : C, 36.60; H, 3.73; N, 21.34. Found: C, 37.07; H, 3.96; N, 21.19.

#### D. Reagent Solutions

(1) Acetazolamide Reagent - 2-Acetyl-amino-1,3,4-thiadiazole-5-sulfonamide (1.6 mg) was dissolved in several drops of dimethylsulfoxide and diluted to 50 ml with water. Further dilutions were with water.

(2) Biotin- and DNP-Modulator Reagents - The biotin-modulator conjugate (2.0 mg) and the DNP-modulator conjugate (1.7 mg) were each dissolved in 10 ml 0.1 M sodium carbonate buffer, pH 10.5. Further dilutions were with water.

(3) Avidin Reagent - Lyophilized avidin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 10 mM Tris-HCl buffer, pH 7.4, at a level of 10.5 activity units per ml (one unit = amount capable of binding 1  $\mu$ g of biotin).

(4) Antibody to Dinitrophenyl (DNP) Reagent - Antisera raised against DNP was chromatographed at 4° C. on a 5 x 70 cm column of Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) with 0.1 M Tris-HCl buffer, pH 8.2, containing 1 M sodium chloride. The second eluted peak determined by optical density monitored at 280 nm contained the antibody activity to DNP.

(5) p-Nitrophenyl Acetate Reagent - 5 mg in 0.3 ml acetone adjusted to 10 ml with distilled water with stirring.

### EXAMPLE 2

#### Esterase Activities of Carbonic Anhydrase as a Function of Acetazolamide Concentration

Carbonic anhydrase, 0.013 International Units, was incubated for 5 min at room temperature in a series of 0.66 ml aqueous solutions containing, respectively, 100  $\mu$ l 0.1 M diethylmalonic buffer, pH 7.4, and the concentrations of acetazolamide and avidin given in Table 1 (concentrations of acetazolamide and avidin based on 1 ml final volume). p-Nitrophenyl Acetate Reagent (0.33 ml) was added to each solution to give a total volume of 1 ml. The hydrolysis rate for p-nitrophenyl acetate was determined by monitoring absorbance at 348 nm.

The results, expressed as the average of duplicate measurements, are presented in Table 1.

Table 1

Acetazolamide Concentration ( $\mu$ M)	Avidin (units/ml)	Hydrolysis Rate ( $\Delta OD_{348}/\text{min}/\text{ml}$ )
0.000	—	0.136
0.144	—	0.088
0.288	—	0.047
0.144	0.105	0.089
0.000	—	0.111
0.000	0.105	0.106

These data indicate that acetazolamide inhibited the enzyme carbonic anhydrase and that the presence of avidin had no significant effect on such inhibition.

### EXAMPLE 3

#### Esterase Activities of Carbonic Anhydrase as a Function of Biotin-Modulator Conjugate Concentration

Carbonic anhydrase, 0.027 International Units, was incubated for 5 min at room temperature in a series of 0.66 ml aqueous solutions containing 100  $\mu$ l 0.1 M diethylmalonic buffer, pH 7.4, and the concentrations of the biotin-modulator conjugate (prepared according to Example 1, Part B) indicated in Table 2 (concentration of conjugate based on 1 ml final volume). Esterase activity, as measured by hydrolysis rate, was determined for each reaction mixture as in Example 2. The results are presented in Table 2 and in graphical form in FIG. 1 of the drawing.

Table 2

Biotin-Modulator Conjugate Concentration ( $\mu$ M)	Hydrolysis Rate ( $\Delta OD_{348}/\text{min}/\text{ml}$ )
0.00	0.136
0.05	0.120
0.10	0.109
0.20	0.103
0.30	0.071
0.40	0.057
0.50	0.040

These data indicate that the biotin-modulator conjugate possesses useful inhibitory properties relative to carbonic anhydrase and is slightly less efficient than the underivatized inhibitor, acetazolamide.

### EXAMPLE 4

#### Direct Binding Assay for Avidin; Competitive Binding Assay for Biotin; Use of Derivatized Acetazolamide as Labeling Substance

A series of binding reaction mixtures were prepared, each to a volume of 0.66 ml and containing 100  $\mu$ l 0.1 M diethylmalonic buffer, pH 7.4, and the concentrations of biotin-modulator conjugate (prepared as in Example 1, Part B), biotin, and avidin indicated in Table 3 (indicated concentrations based on 1 ml final volume). After a 5 min incubation at room temperature, 0.027 International Units carbonic anhydrase was added to each reaction mixture followed by an additional 5 min incubation. p-Nitrophenyl Acetate Reagent (0.33 ml) was then added to each and the rate of hydrolysis measured by monitoring absorbance at 348 nm.

The results, expressed as the average of duplicate measurements, are presented in Table 3. The results of reaction mixtures 2 through 9 are presented in graphical form in FIG. 2 of the drawing.

The results of reaction mixtures 2 through 9 demonstrate the effect of various levels of avidin on the inhibitory activity of biotin-modulator conjugate on esterase activities of carbonic anhydrase. For a constant level of biotin-modulator conjugate, the rate of enzymatic hydrolysis is directly related to the amount of avidin present. The results of reaction mixtures 8, 10, and 11 show that when biotin is present, the rate of hydrolysis is inversely related to the amount present.

Table 3

Reaction Mixture	Biotin-Modulator Conjugate ( $\mu$ M)	Biotin ( $\mu$ M)	Avidin (units/ml)	Rate of Hydrolysis
1	—	—	—	0.128
2	0.50	—	0.011	0.049
3	0.50	—	0.026	0.056
4	0.50	—	0.040	0.066
5	0.50	—	0.053	0.075



the improvement wherein said labeling substance is a reversibly binding enzyme inhibitor and said means comprises additionally an enzyme whose activity is affected by said enzyme inhibitor, the binding constant for association of said inhibitor and said enzyme being less than about  $10^{11}$  molar<sup>-1</sup>.

10. Means as claimed in claim 9 wherein the binding constant for the association of said reversibly binding enzyme inhibitor and said enzyme is between about  $10^9$  and about  $10^{10}$  molar<sup>-1</sup>.

11. Means as claimed in claim 9 wherein said reversibly binding enzyme inhibitor is a competitive inhibitor of the enzyme.

12. Means as claimed in claim 11 wherein said reversibly binding competitive inhibitor and the enzyme inhibited thereby are, respectively,

- (a) acetazolamide, or an effective analog thereof, and carbonic anhydrase;
- (b) sulfanilamide, or an effective analog thereof, and carbonic anhydrase;
- (c) phenyl trimethylammonium ion, or an effective analog thereof, and acetylcholinesterase;
- (d) saccharo-1,4-lactone, or an effective analog thereof, and  $\beta$ -glucuronidase;
- (e) 4-amino-10-methyl-pteroylglutamic acid, or an effective analog thereof, and dihydrofolate reductase; or
- (f) 2,6,8-trichloropurine, or an effective analog thereof, and uricase.

13. Means as claimed in claim 9 wherein said enzyme inhibitor, as a component of said conjugate, affects the activity of said enzyme differently when said conjugate is in said bound-species than when in said free-species,

said means comprising (i) said conjugate wherein said binding component is said ligand, a specific binding analog of said ligand, or a specific binding partner of said ligand, and (ii) if said binding component is said ligand or a specific binding analog thereof, a specific binding partner of said ligand.

14. Means as in claim 9 which comprises (i) said conjugate wherein said binding component is said ligand or a specific binding analog of said ligand, and (ii) a specific binding partner of said ligand.

15. Means as in claim 14 wherein one of said conjugate and said specific binding partner is in a form which is insoluble in said liquid medium.

16. Means as in claim 9 which comprises (i) said conjugate in a form which is soluble in said liquid medium and wherein said binding component is a specific binding partner of said ligand, and (ii) said ligand or a specific binding analog thereof in a form which is insoluble in said liquid medium.

17. Means as in claim 9 which comprises (i) said conjugate wherein said binding component is a specific binding partner of said ligand, and (ii) a specific binding partner of said ligand.

18. Means as in claim 17 wherein one of said conjugate and said specific binding partner is in a form which is insoluble in said liquid medium.

19. Means as in claim 9 wherein said ligand is selected from the group consisting of antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites, and pharmacological agents, and their receptors and binding substances.

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